

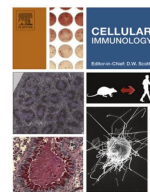
# NF $\kappa$ B and TGF $\beta$ contribute to the expression of PTPN3 in activated human lymphocytes

中山, 和典

<https://hdl.handle.net/2324/4474986>

---

出版情報 : Kyushu University, 2020, 博士 (医学), 課程博士  
バージョン :  
権利関係 : (c) 2020 Elsevier Inc. All rights reserved.



## NFκB and TGFβ contribute to the expression of PTPN3 in activated human lymphocytes

Kazunori Nakayama<sup>a</sup>, Hideya Onishi<sup>a,\*</sup>, Akiko Fujimura<sup>a</sup>, Akira Imaizumi<sup>a</sup>, Makoto Kawamoto<sup>a</sup>, Yasuhiro Oyama<sup>a</sup>, Shu Ichimiya<sup>a</sup>, Satoko Koga<sup>a</sup>, Yuichi Fujimoto<sup>b</sup>, Kinichi Nakashima<sup>b</sup>, Masafumi Nakamura<sup>c</sup>

<sup>a</sup> Department of Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

<sup>b</sup> Department of Stem Cell Biology and Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

<sup>c</sup> Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

### ARTICLE INFO

#### Keywords:

PTPN3  
NFκB  
TGFβ  
Activated lymphocyte  
Immunotherapy

### ABSTRACT

We previously reported that protein tyrosine phosphatase non-receptor type 3 (PTPN3), which is upregulated in activated lymphocytes, acts as an immune checkpoint. However, the mechanism by which PTPN3 expression is enhanced in activated lymphocytes is unknown. In this study, we analyzed the mechanism of PTPN3 expression in activated lymphocytes with a view for developing a novel immune checkpoint inhibitor that suppresses PTPN3. Through the activation process, lymphocytes showed enhanced NFκB activation as well as increased PTPN3 expression. NFκB enhanced proliferation, migration, and cytotoxicity of lymphocytes. Furthermore, NFκB enhanced PTPN3 expression and tyrosine kinase activation. TGFβ reduced PTPN3 expression and NFκB activation in the cancer microenvironment, and suppressed the biological activity of lymphocytes. The results of this study are expected to provide significant implications for improving existing immunotherapy and developing novel immunotherapy.

### 1. Introduction

Surgical therapy, chemotherapy, and radiotherapy have played a central role in cancer treatment. In recent years, immunotherapy has received increased attention as a novel treatment for cancer. Among immunotherapies, immune checkpoint inhibitors have shown efficacy in patients that do not respond to other treatments [1,2]. However, the effectiveness of immune checkpoint inhibitors is still limited, and these treatments are associated with side effects such as autoimmune diseases and high cost. Therefore, the development of new cancer immune therapies is an important focus of research.

Activated lymphocytes are mainly responsible for the anti-tumor effect of cancer immunotherapy [3]. Through the process of lymphocyte activation, the proliferation, migration, and cytotoxicity activities of lymphocytes are enhanced [4]. Protein tyrosine phosphatase (PTP) is an enzyme that dephosphorylates tyrosyl residues of tyrosine and counteracts the actions of protein tyrosine kinase (PTK), which is involved in cell proliferation, differentiation, adhesion, migration, and

other activities through its phosphorylation of tyrosyl residues [5,6]. We previously reported that protein tyrosine phosphatase non-receptor type 3 (PTPN3) exhibits inhibitory actions on activated lymphocytes as an immune checkpoint [7]. Lymphocytes are exposed to antigen presentation from antigen presenting cells and gain cytotoxic activity. At that time, stimulation from TCR/CD3 is transmitted inside the cell, and the NFκB transcription factor is activated through a signal cascade [8]. However, the relationship between PTPN3 expression and NFκB in the process of lymphocyte activation has not been reported and the PTPN3 expression mechanism in activated lymphocytes is not clear. Elucidating the mechanism of the enhanced expression of PTPN3 in activated lymphocytes may lead to the development of more effective immune therapies.

In this study, we focused on the relationship between NFκB and PTPN3 expression and examined the mechanism of enhanced PTPN3 expression in activated lymphocytes. These results may lead to the improvement of immune therapeutic effects or the development of novel immunotherapies.

\* Corresponding author at: Department of Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.

E-mail address: [ohnishi@surg1.med.kyushu-u.ac.jp](mailto:ohnishi@surg1.med.kyushu-u.ac.jp) (H. Onishi).

<https://doi.org/10.1016/j.cellimm.2020.104237>

Received 5 June 2020; Received in revised form 7 October 2020; Accepted 8 October 2020

Available online 17 October 2020

0008-8749/© 2020 Elsevier Inc. All rights reserved.

## 2. Materials and methods

### 2.1. Activation of lymphocytes

Peripheral blood mononuclear cells (PBMCs) were collected from heparinized peripheral blood of healthy volunteers via HISTOPAQUE-1077 (Merck KGaA, Germany) density gradient centrifugation. PBMCs were cultured in RPMI-1640 (Nacalai Tesque, Japan) supplemented with 0.5% human serum, 2000 units/ml penicillin (Meijiseika, Japan), 10 µg/ml streptomycin (Meijiseika), and 200 U/ml IL-2 (Primmune, USA) in a six-well plate coated with 2.5 µg/ml anti-CD3 monoclonal antibody (OKT3, JANSSEN PHARMACEUTICAL K.K., Japan). Non-adherent fractions were collected as activated lymphocytes.

Informed consent was obtained from all individuals, and all experiments were approved by the Kyushu University Ethics Committee (29-251 and 28-277).

### 2.2. Cell viability and treatments

Cell viability was measured by the Trypan blue dye exclusion assay (Sigma Aldrich, USA). Ammonium pyrrolidinedithiocarbamate (PDTTC), an inhibitor of NFκB nuclear translocation (Sigma Chemical, Germany), was used in some experiments.

### 2.3. Cancer cell culture

The human pancreatic ductal adenocarcinoma cell lines SUIT2, PANC1, and AsPC1 (American Type Culture Collection, USA) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Thermo Fisher Scientific, USA) and antibiotics (2000 units/ml penicillin and 10 µg/ml streptomycin).

### 2.4. Lymphocyte-cancer cell co-culture

Transwell inserts (pore size, 0.4 µm; BD Biosciences, USA) were used for co-culture of lymphocytes and cancer cells. The upper chamber included PBMCs ( $1 \times 10^6$  cells/ml) from healthy donors, and the lower chamber included pancreatic ductal adenocarcinoma cell lines ( $1 \times 10^5$  cells/ml). After 24 h or 72 h co-incubation, lymphocytes were analyzed.

### 2.5. Preparation of cancer cell culture supernatant

Pancreatic cancer cells ( $2 \times 10^5$  cells/well) were seeded into a 6-well plate and incubated for 72 h. After the incubation time, the supernatant was centrifuged and filter sterilized (0.22 µm).

### 2.6. RNA interference

The pLLX vector was kindly provided by Dr. Z. Zhou (University of Pennsylvania School of Medicine) and Dr. M. E. Greenberg (Harvard Medical School). The nucleotide sequence for short hairpin RNA (shRNA) targeting NFκB p65 was as follows: shNFκB p65, GGGATGAGATCTTCCTACTGTGTGA. The oligonucleotide was ligated into the pLLX vector. This plasmid vector was co-transfected with the pCMV-VSV-G-RSV-Rev plasmid and pCAG-HIVgp plasmid (Riken, Japan) using Lipofectamine 2000 reagent (Invitrogen, USA) into the 293 T cell line to produce lentiviral stock. Nucleotide sequences for shRNA targeting PTPN3 were as follows: shPTPN3 #1, CAATCAGAAGCA GAATCCTGCTATA, and shPTPN3 #2, GACAGCTACTTAGTCTTGATCCGTA. The oligonucleotides were ligated into pcDNA<sup>TM</sup>6.2-GW/Em-miR (#K4934-00; Thermo Fisher Scientific). The miR-RNAi cassette was transferred from pcDNA<sup>TM</sup>6.2-GW/Em-miR into the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector using MultiSite Gateway® technologies (Thermo Fisher Scientific). This plasmid vector was then co-transfected with the ViraPower<sup>TM</sup> Packaging Mix (included in BLOCK-iT<sup>TM</sup> Lentiviral Poll 1 miR RNAi Expression Systems; Thermo

Fisher Scientific) using Lipofectamine 2000 reagent into the 293FT cell line to produce the lentiviral stock. Lentiviral infection of lymphocytes was performed from culture day 2.

### 2.7. Real-time RT-PCR

Total RNA was extracted using a High Pure RNA Isolation kit (Roche, Switzerland) and quantified by spectrophotometry (NanoDrop 1000; Thermo Scientific). RNA (1.0 µg) was reverse transcribed to cDNA with the Verso cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Reactions were run on a 7500 Real-Time PCR System (Applied Biosystems, USA) using PowerUp SYBR Green Master Mix (Applied Biosystems) [9]. Human *PTPN3* real-time primer was purchased from Bio-Rad. Sequences of PCR primers are as follows: *β-actin* forward, 5'-TTGTTACAGGAAGTCCCTTGCC-3'; reverse, 5'-ATGCTATCACCTCCCCTGTGTG-3'; and *NFκB p65* forward, 5'-CGCTGCA TCCACAGTTTCCA-3'; reverse, 5'-AGGGGTTGTGTGGTCTGG-3'. The amount of each target gene in each sample was normalized to the level of *β-actin* in that sample.

### 2.8. Western blotting

Western blotting was performed as described previously [10]. In some experiments, pancreatic cancer supernatants, recombinant TGFβ1 (No. 209-16544, Wako, Japan), anti TGFβ1 neutralizing Ab (#521707, BioLegend, USA), and control mouse IgG Ab (#400165, BioLegend) were added to the culture and were incubated at the indicated period. 50 µg of protein was used for assay. Blots were incubated with anti-PTPN3 (1:200, sc-9789; Santa Cruz Biotechnology, USA), anti-ZAP (1:2000, No. 3165; Cell Signaling Technology), anti-pZAP (1:1000, No. 2701; Cell Signaling Technology), anti-LCK (1:1000, No. 2657; Cell Signaling Technology), anti-pSRC (1:1000, No. 2101; Cell Signaling Technology), anti-SMAD2/3 (0.5 µg/ml, AF3797-SP; R&D systems, USA), anti-pSMAD2/3 (2 µg/ml, MAB8935-SP; R&D systems) or anti-α tubulin antibodies (1:1000; Sigma Aldrich, USA) overnight at 4 °C. The blots were then incubated with HRP-linked secondary antibody (1:10000; GE Healthcare, UK) at room temperature for 1 h. Immuno-complexes were detected with Chemi-Lumi One Super (Nacalai Tesque) and visualized with EZ Capture ST (ATTO, Japan). To normalize protein bands, α-tubulin was used as a protein loading control.

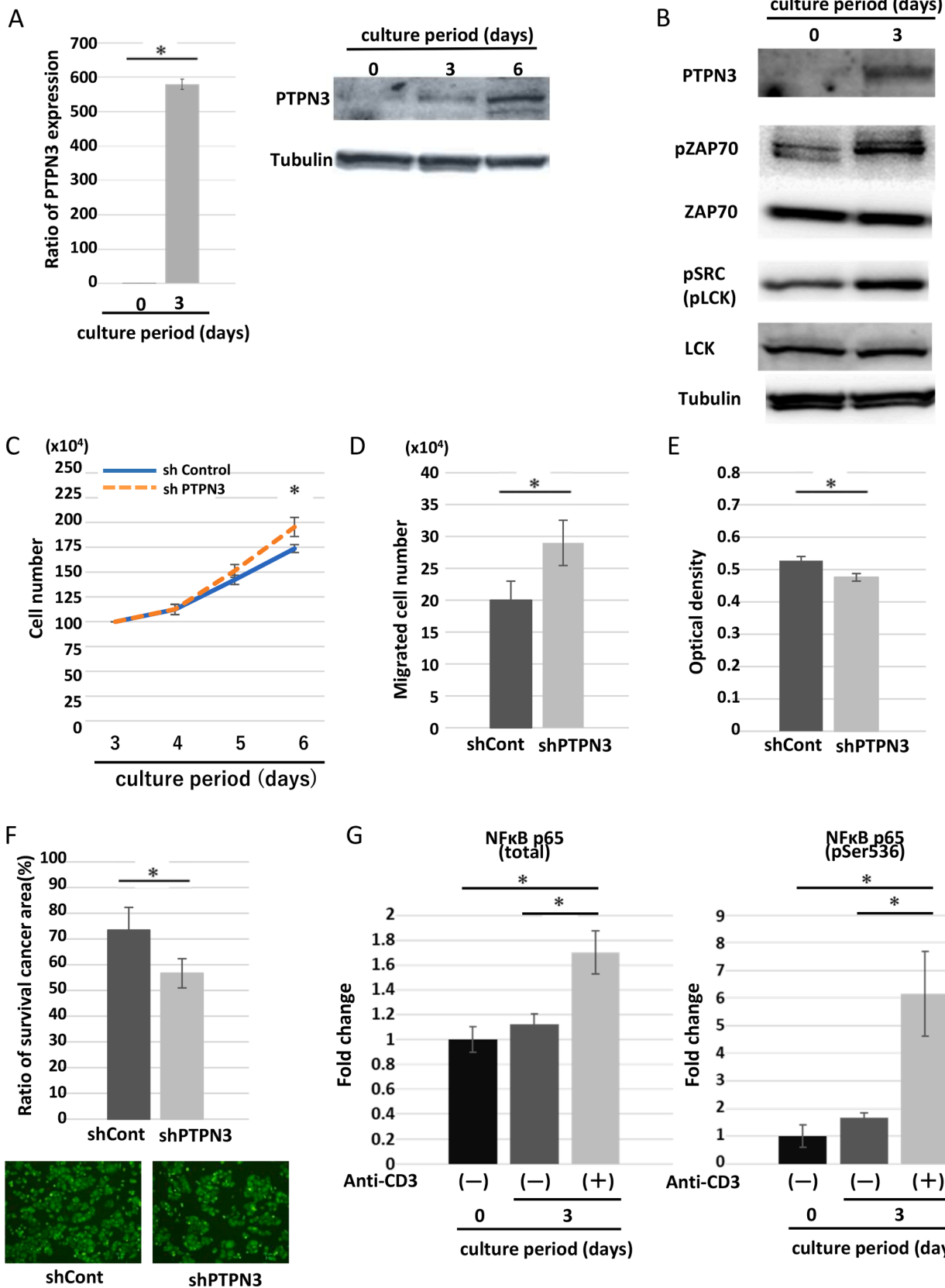
### 2.9. Migration assay

Migration ability of lymphocytes was evaluated in migration assays using Transwell inserts (pore size, 3.0 µm; BD Biosciences, USA). Lymphocytes ( $1 \times 10^5$  cells) were added to the upper chamber and incubated for 16 h. After incubation, all cells that had migrated from the upper to the lower side of the filter were counted using a light microscope (BX50; Olympus, Japan).

### 2.10. Cytotoxic assay

Target SUIT2 or PANC1 cells ( $1 \times 10^4$  cells/well) were seeded into a 96-well flat-bottom plate and incubated for 12 h to allow adherence. Effector cells (activated lymphocytes) were then added to the culture. Target and effector cells were co-incubated for 72 h at effector/target cell ratios of 5:1, 10:1 and 20:1. To quantify viable adherent cancer cells, the Cell Count Reagent SF (Nacalai Tesque) was added to the wells for 1 h at 37 °C. The absorbance at 492 nm was then measured using a microplate reader (Biotrak visible plate reader; Amersham Biosciences, USA). Viable cancer cells were detected by subtracting the absorbance of lymphocytes alone from that of under co-culture conditions. Optical density indicates the calculated absorbance of viable cancer cells [11].

To confirm the cytotoxicity of activated lymphocytes, lymphocytes and calcein-labeled cancer cells (PANC1 or SUIT2) were co-cultured at 20:1 (E:T) ratio. After co-culture, ratio of survival cancer area was



(caption on next page)

**Fig. 1.** PTPN3 expression, tyrosine kinase activity, and NF $\kappa$ B activity were increased in activated lymphocytes. (A) PBMCs from healthy volunteers were activated by anti-CD3 antibody and IL-2. Left, PTPN3 mRNA expressions in lymphocytes before and after activation were estimated by real-time RT-PCR. Right, PTPN3 protein expressions in lymphocytes before and after activation were investigated by western blotting. (B) Western blotting was performed for the indicated proteins in lymphocytes before and after activation. (C) Activated lymphocytes were infected with shPTPN3 or shControl lentivirus at day 2. Cell numbers were counted using a light microscope. (D) Activated lymphocytes were infected with shPTPN3 or shControl lentivirus at day 2 and incubated for 48 h. The migration ability of lymphocytes at day 5 was analyzed. (E) Lymphocytes (day 5) and cancer cells (SUIT2) were co-cultured at 20:1 (E:T) ratio and the cytotoxicity of lymphocytes was analyzed. (F) Lymphocytes and calcein-labeled cancer cells (SUIT2) were co-cultured at 20:1 (E:T) ratio. After co-culture of 8 h, ratio of survival cancer area was corrected in the area of where only the cancer cells were cultured. (G) Total and phosphorylated NF $\kappa$ B p65 were measured in lymphocytes (day 0) and after incubated with or without anti-CD3 antibody (day 3) by ELISA. Data are calibrated to the lymphocytes (day0). The lymphocytes used in each experiment of (A-G) were obtained from three different healthy volunteers. Similar results were obtained in all experiments. Data are presented as the mean  $\pm$  SD. \* $p < 0.05$ .

corrected in the area of where only the cancer cells were cultured. Calcein-labeled survival cancer cells area was measured by the ImageJ software program.

### 2.11. Fluorescence-activated cell sorting (FACS)

Cells were stained with phycoerythrin (PE)-conjugated anti-CD3 mAbs (BD Pharmingen, USA). Mouse IgG was used as an isotype control (BD Pharmingen). The fluorescence intensity or percent positive cells were measured using an FACS Calibur flow cytometer (BD Pharmingen) and analyzed with the CELL Quest software program (BD Pharmingen).

### 2.12. Enzyme-linked immunosorbent assay (ELISA)

To evaluate NF $\kappa$ B p65, lymphocytes ( $5 \times 10^4$  cells/well) were lysed, and total and phosphorylated (Ser536) NF $\kappa$ B p65 were measured using a NF $\kappa$ B p65 (Total/Phospho) Human InstantOne™ ELISA Kit (Thermo Fisher Scientific), according to the manufacturer's protocol.

To evaluate TGF $\beta$ 1, tumor cells were seeded into a 6-well plate. After 48 h of incubation, supernatants were collected, and the concentration of TGF $\beta$ 1 was measured using the Human TGF $\beta$ 1 ELISA Kit (Proteintech, USA) according to the manufacturer's protocol.

### 2.13. PCR amplification and cloning of the PTPN3 promoter

Genomic DNA was isolated from healthy human PBMCs using a Monofas DNA Kit IV (GL Science, Japan) and quantified. Genomic DNA was used to PCR amplify PTPN3 promoter sequences to amplify region A (-1841 bp to -649 bp w.r.t. TSS + 1) using primers (forward, 5'-ATTGCCACCGCATGGTACCTCACTCAACTCCACTCGGG-3'; reverse, 5'-ATTGCTAGCTCCTGACCACCTTCCTCGAT-3') flanked with Kpn I and Nhe I sites. Region B (-170 bp to +451 bp w.r.t. TSS + 1 in the PTPN3 promoter) was created by artificial gene synthesis (Invitrogen). PCR was performed using the KOD One PCR Master Mix (TOYOBO, Japan). The amplified products were subjected to agarose gel electrophoresis, and the amplicons were excised from the gel, purified and cloned in the pGL3-Promoter vector (Promega, USA) and designated as pGL3 (PTPN3-A) and pGL3 (PTPN3-B). The vector was transfected in DH5 $\alpha$  cells, which were plated and incubated. Positive colonies were selected, and the sequence of the cloned vectors were confirmed by sequencing using BigDye® Terminator v3.1 (Applied Biosystem) on the Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystem).

### 2.14. Dual-luciferase assay

For luciferase assays, 293 T cells were plated in 12-well plates and cultured overnight. The cells were then transfected with the reporter gene construct (pGL3 (PTPN3-A) or pGL3 (PTPN3-B)), pHRV-SV40, and expression plasmid (pCMV4-p65 expression plasmid or pCMV4-control plasmid; Addgene, USA) using Lipofectamine 2000 reagent. After 48 h, luciferase activity was analyzed using a dual-luciferase assay kit (Promega) following the manufacturer's protocol.

### 2.15. Statistical analyses

Data are presented as the means  $\pm$  standard deviation (SD). Student's *t*-test was used to compare continuous variables between two groups. *P*-values of  $< 0.05$  were considered statistically significant.

## 3. Results

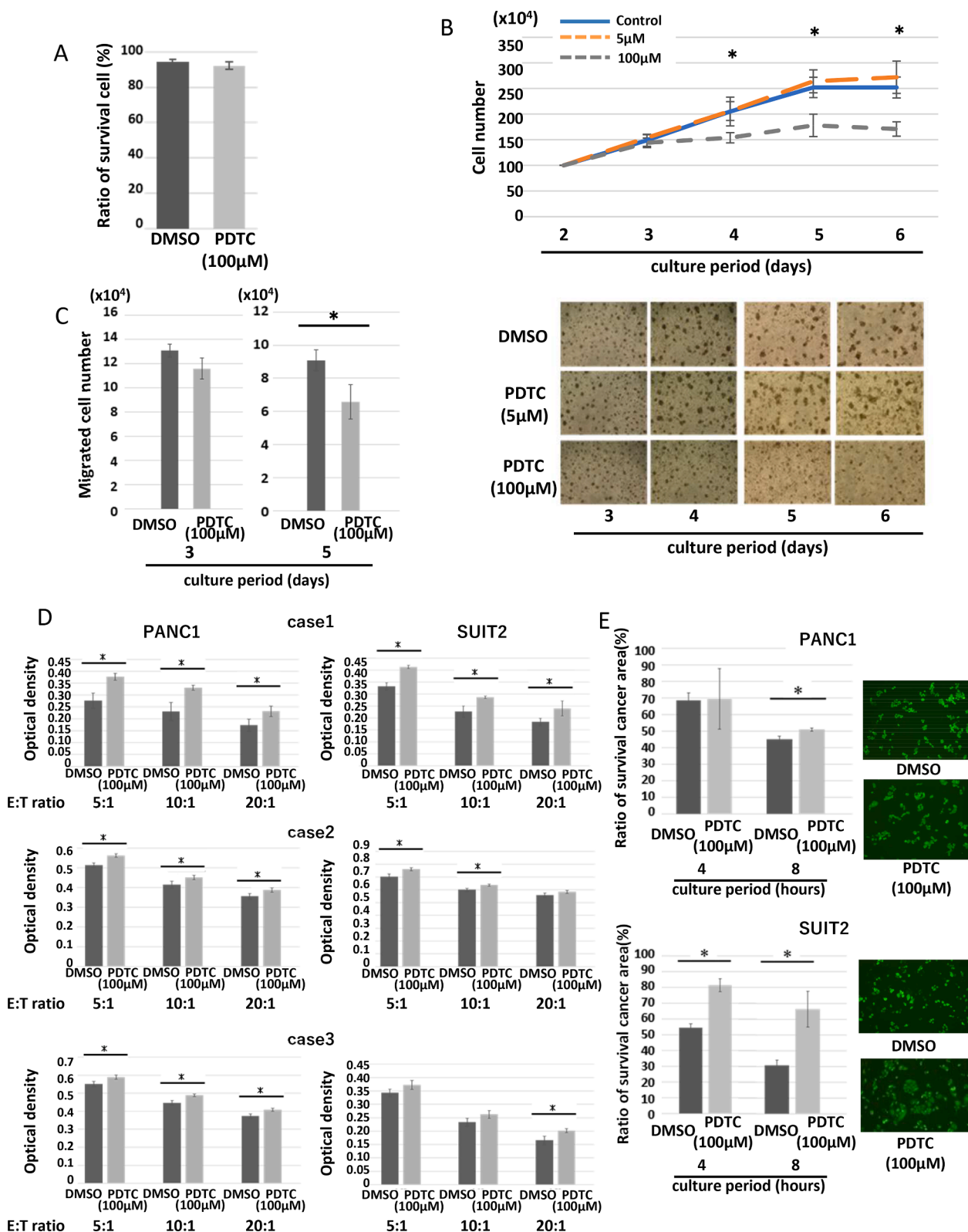
### 3.1. PTPN3 expression, tyrosine kinase activity, and NF $\kappa$ B activity were enhanced in lymphocytes activated with anti-CD3 antibody and IL-2

We first examined PTPN3 expression, tyrosine kinase activation, and NF $\kappa$ B activation in activated lymphocytes. PBMCs from healthy human peripheral blood were separated and activated using anti-CD3 antibodies and IL-2 as described in the Methods [7]. PTPN3 mRNA and protein expressions were very low in lymphocytes before activation, but increased expression was observed through the activation process (Fig. 1A). TCR/CD3 signaling is important for PTPN3 expression, and increased phosphorylation of ZAP70, the major tyrosine kinase downstream of TCR/CD3, and phosphorylation of SRC (LCK), an important tyrosine kinase upstream of TCR and downstream of CD4/CD8, were also observed in activated lymphocytes (Fig. 1B) [7]. The function of activated lymphocytes such as proliferation (Fig. 1C and Supplementary Fig. S1A), migration (Fig. 1D and Supplementary Fig. S1B) and cytotoxicity (Fig. 1E, F and Supplementary Fig. S1C) augmented significantly when PTPN3 was inhibited in activated lymphocytes. In addition, NF $\kappa$ B activity was enhanced with the activation of lymphocytes, and stimulation from anti-CD3Ab was also important for the activation of NF $\kappa$ B in lymphocytes (Fig. 1G). Together these results suggest that PTPN3 expression, tyrosine kinase activity, and NF $\kappa$ B activity were enhanced in activated lymphocytes.

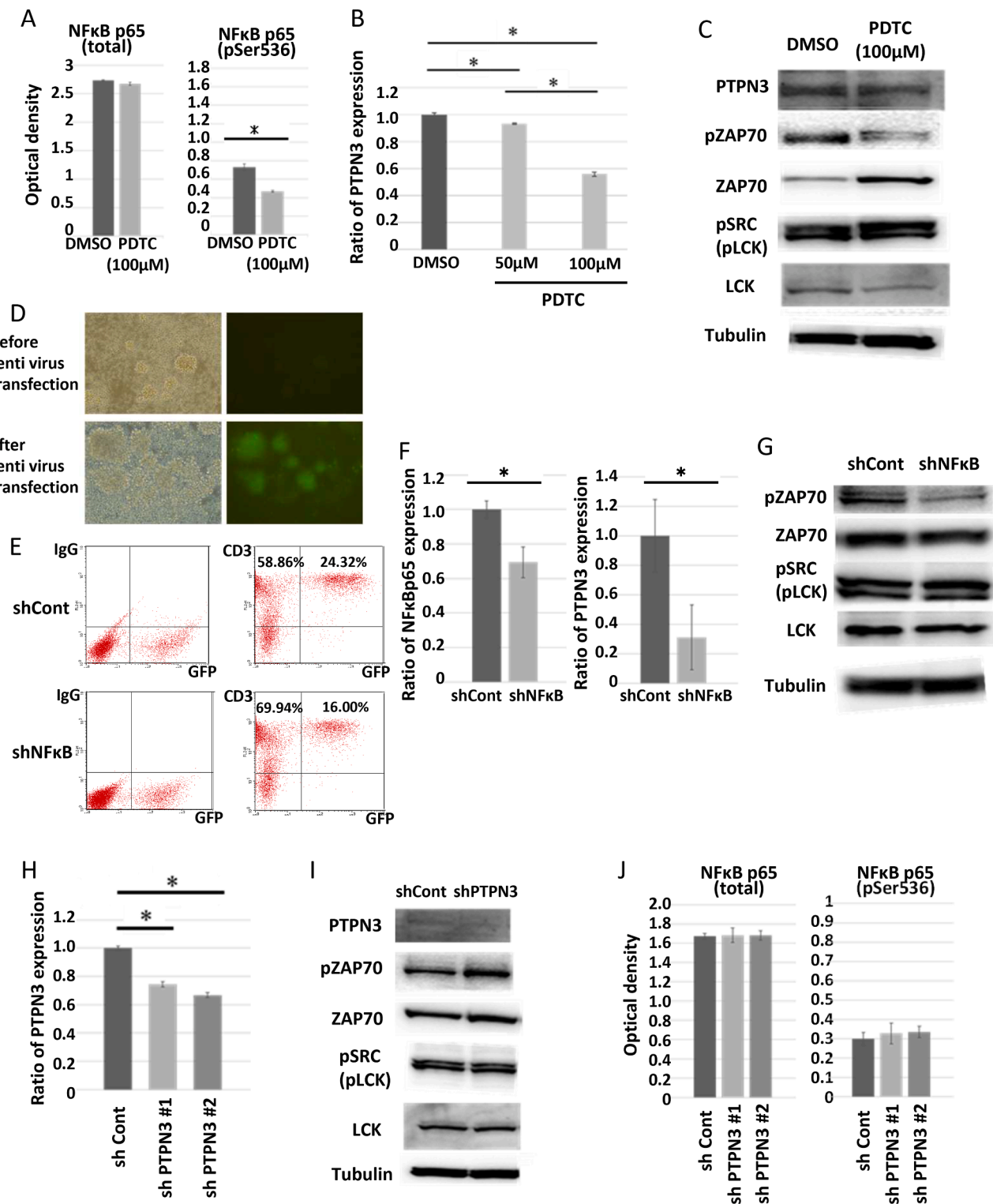
### 3.2. NF $\kappa$ B was involved in the proliferation, migration, and cytotoxicity of lymphocytes

To investigate whether NF $\kappa$ B is important for lymphocyte function, PDTC was administered before or after lymphocyte activation [12–14]. The p65 and p50 NF $\kappa$ B subunits form a complex in the cytoplasm, and I $\kappa$ B binding to the complex leads to NF $\kappa$ B inactivation in the cytoplasm. Reactive oxygen species (ROS) is thought to be involved in the process leading to the release of I $\kappa$ B and subsequent NF $\kappa$ B nuclear translocation and activation. PDTC acts as an antioxidant and neutralizes ROS, which prevents the release of I $\kappa$ B and inhibits the nuclear translocation of NF $\kappa$ B.

First, PBMCs were activated and then PDTC was used to suppress NF $\kappa$ B activation. We confirmed that 100  $\mu$ M of PDTC did not affect the viability in activated lymphocytes (Fig. 2A). We examined the proliferation of activated lymphocytes treated with PDTC. No change was observed in the group treated with PDTC at a low concentration of 5  $\mu$ M, but inhibition of proliferation was observed at concentrations of 100  $\mu$ M (Fig. 2B). In addition, suppression of activated lymphocyte migration ability was observed in the PDTC-treated group compared with controls (Fig. 2C). We further evaluated the cytotoxicity of lymphocytes by co-culture of lymphocytes with pancreatic cancer cells (SUIT2 and PANC1) in two different methods. A decrease in cytotoxicity was



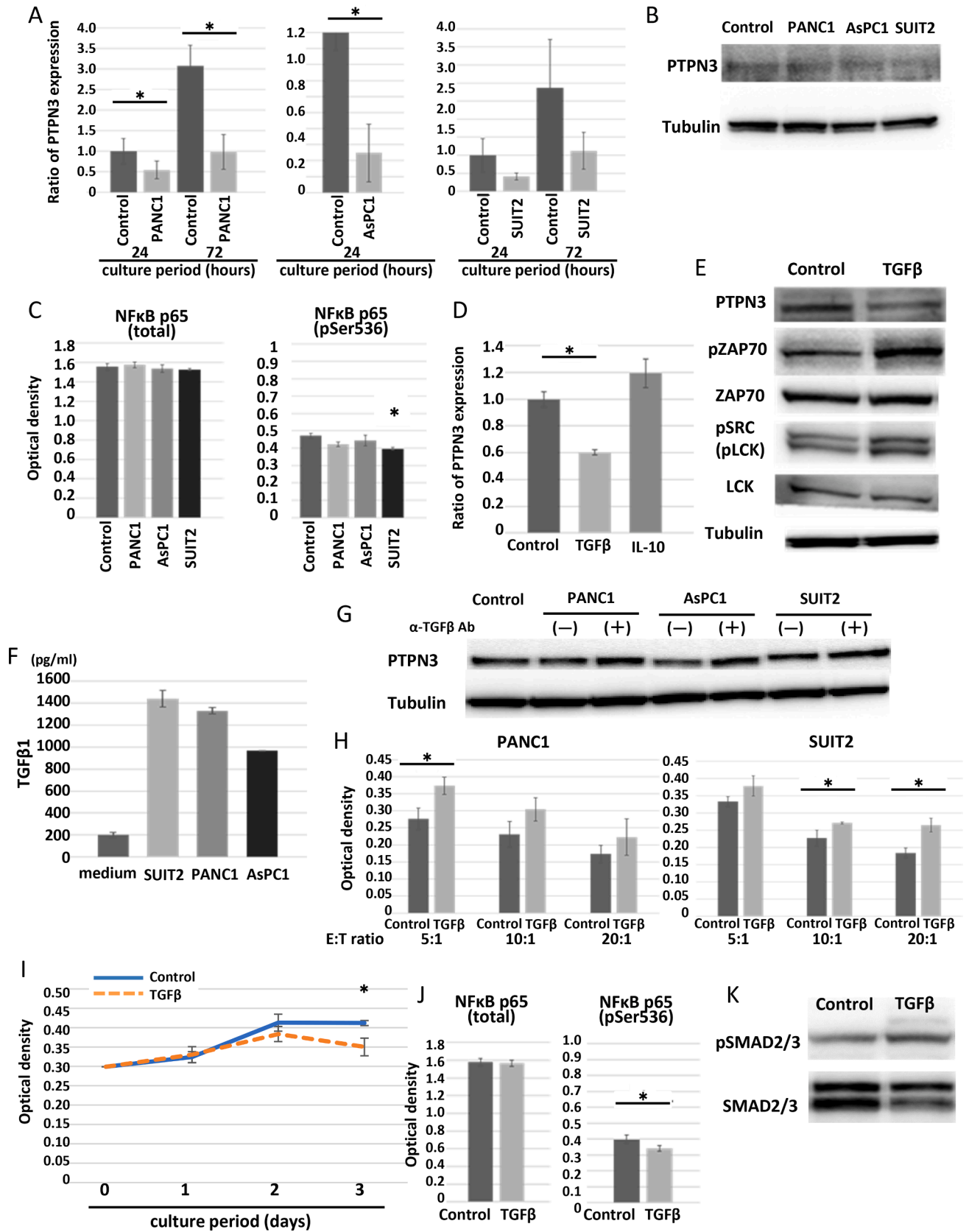
**Fig. 2.** NFκB suppression inhibited the functions of activated lymphocytes. (A) Activated lymphocytes were treated with 100 µM ammonium pyrrolidinedithiocarbamate (PDTC) or DMSO (as control) at day 2. Viability of activated lymphocytes was investigated by the count of viable cells by trypan blue staining method at day 3. (B) Activated lymphocytes were treated with 5 µM or 100 µM PDTC or DMSO (as control) at day 2. Cell numbers were counted using a light microscope. Representative pictures are shown. Original magnification ×20. (C) Lymphocytes were treated with 100 µM PDTC or DMSO. The migration ability of lymphocytes at day 3 or day 5 was analyzed. (D) Lymphocytes (day 3) and cancer cells (PANC1 or SUI2) were co-cultured at various ratios and the cytotoxicity of lymphocytes was analyzed. Data are representative of three independent healthy volunteers experiments. (E) Lymphocytes and calcein-labeled cancer cells (PANC1 or SUI2) were co-cultured at 20:1 (E:T) ratio. After co-culture of indicated time, ratio of survival cancer area was corrected in the area of where only the cancer cells were cultured. Representative images are shown. Original magnification ×20. Data are presented as mean ± SD. \*p < 0.05.



**Fig. 3.** NFκB suppression inhibits PTPN3 expression and tyrosine kinase expression. Activated lymphocytes were treated with 100 μM PDTC or DMSO (as control) at day 2. (A) Total and phosphorylated NFκB p65 expression in lymphocytes at day 3 were measured by ELISA. (B) PTPN3 mRNA expression in activated lymphocytes treated with 50 μM, 100 μM PDTC or DMSO were estimated by real-time RT-PCR. (C) Western blotting was performed for the indicated proteins in activated lymphocytes treated with PDTC or DMSO. (D,E) PBMCs were infected with shNFκB p65 or shControl lentivirus at day 2 and incubated for 48 h. GFP expression in lymphocytes was examined and the percentage of CD3 (+), GFP (+) lymphocytes was measured by FACS. (F) NFκB p65 and PTPN3 mRNA expressions were estimated by real-time RT-PCR. (G) Western blotting was performed for the indicated proteins in activated lymphocytes treated as indicated. (H) PBMCs were infected with shPTPN3 or shControl lentivirus at day 2 and incubated for 48 h. PTPN3 mRNA expression in lymphocytes was investigated by real-time RT-PCR. (I) Western blotting was performed for the indicated proteins in activated lymphocytes treated as indicated. (J) Total and phosphorylated NFκB p65 in lymphocytes were measured by ELISA. Data are presented as mean ± SD. \*p < 0.05.







(caption on next page)

**Fig. 5.** Cancer cell culture supernatant and TGF $\beta$  affect PTPN3 expression and NF $\kappa$ B activity in lymphocytes. Co-culture experiments were performed using Transwell inserts, pancreatic ductal adenocarcinoma cell lines were cultured in the lower chamber overnight and then activated lymphocytes were added in the upper chamber and incubated for 24 h or 72 h. (A) PTPN3 mRNA and protein expression of lymphocytes were estimated by real-time RT-PCR and (B) western blotting. (C) NF $\kappa$ B expression and activation of lymphocytes were measured by ELISA with triplicate-well. (D) TGF $\beta$  (2 ng/ml) or IL-10 (20 ng/ml) was added to activated lymphocytes at day 2 and cells were incubated for 72 h. PTPN3 mRNA expression of lymphocytes were estimated by real-time RT-PCR. (E) Western blot of the indicated proteins was performed. (F) TGF $\beta$  in pancreatic cancer cell culture supernatant was measured by ELISA with triplicate-well. (G) Cancer cell culture supernatant were incubated with neutralizing mAb against TGF $\beta$ 1 (10 ng/ml) or control IgG for 1 h. Then, activated lymphocytes at day 2 were cultured with 50% of the cancer cell culture supernatant for 72 h. PTPN3 protein expression of lymphocytes were estimated by western blotting. (H) The cytotoxicity of lymphocytes was analyzed. Cancer cells (PANC1 or SUIT2) were seeded into a 96-well plate and incubated overnight. Effector cells (activated lymphocytes treated with or without TGF $\beta$ ) were then added to the culture. Target and effector cells were co-cultured for 72 h. After WST reagent solution was added to the well, the absorbance of viable cancer cells was detected by subtracting the absorbance of lymphocytes alone from that of co-culture. OD shows the calculated absorbance of viable cancer cells. (n = 3) (I) The proliferation of activated lymphocytes treated with TGF $\beta$  was determined. Cell numbers were counted using a light microscope. (J) NF $\kappa$ B expression and activation were examined in activated lymphocytes treated with TGF $\beta$  using ELISA with triplicate-well. (K) TGF $\beta$  (2 ng/ml) was added to activated lymphocytes at day 2 and cells were incubated for 24 h. Western blot of the indicated proteins was performed. The lymphocytes used in each experiment of (A-K) were obtained from three different healthy volunteers. Similar results were obtained in all experiments. Data are presented as mean  $\pm$  SD. \*p < 0.05.

observed in the PDTC-treated groups compared with controls (Fig. 2D, E).

We also examined the effects when lymphocytes were activated after suppressing NF $\kappa$ B activation, the proliferation of lymphocytes was unaffected in the group treated with 5  $\mu$ M PDTC, but inhibited by 100  $\mu$ M PDTC (Supplementary Fig. S2A). Suppression of migration was also observed in the PDTC-treated group (Supplementary Fig. S2B). These results suggest that NF $\kappa$ B is involved in the proliferation, migration, and cytotoxicity of activated lymphocytes.

### 3.3. NF $\kappa$ B was involved in PTPN3 expression and tyrosine kinase expression

We next examined the relationship between PTPN3 and NF $\kappa$ B in activated lymphocytes. We suppressed NF $\kappa$ B by two methods: PDTC and shNF $\kappa$ B p65-expressing lentivirus. We confirmed that NF $\kappa$ B p65 nuclear translocation was suppressed by PDTC (Fig. 3A). We found that PTPN3 expression decreased by PDTC treatment in dose dependent manner (Fig. 3B). PTPN3 expression and phosphorylation of ZAP70 were suppressed by PDTC (Fig. 3C). No changes in SRC (LCK) were observed. On the other hand, resting lymphocytes showed low levels of PTPN3 expression and phosphorylation of ZAP70, and PDTC didn't affect PTPN3 expression and phosphorylation of ZAP70 (Supplementary Fig. S2C). We next performed experiments in lymphocytes infected with shControl or shNF $\kappa$ B p65 lentivirus and confirmed the infection efficiency in lymphocytes by expression of GFP (Fig. 3D). GFP-positive cells were mostly CD3<sup>+</sup> positive cells (Fig. 3E). Gene transfection using lentiviral vector is easy to perform in proliferating lymphocytes and CD3<sup>-</sup> cells may be difficult to transfect with lentiviral vectors. We have also previously shown that PTPN3 expression in CD3<sup>+</sup> activated lymphocytes was significantly higher than that in CD3<sup>-</sup> activated lymphocytes, and that lymphocyte activation by PTPN3 inhibition is observed only in activated CD3<sup>+</sup> T cells but not in CD3<sup>-</sup> T cells [7]. We think that difficulty of transfection in CD3<sup>-</sup> cells does not affect our results. After suppression of NF $\kappa$ B in activated lymphocytes by lentivirus, PTPN3 expression and phosphorylation of ZAP70 were both suppressed (Fig. 3F, G). However, upon suppression of PTPN3 in activated lymphocytes by shPTPN3 lentivirus (Fig. 3H, I), NF $\kappa$ B p65 expression and NF $\kappa$ B p65 phosphorylation were not affected (Fig. 3J). Together these results indicate NF $\kappa$ B is involved in the expression of PTPN3 and ZAP70 phosphorylation.

### 3.4. NF $\kappa$ B may be indirectly involved in PTPN3 expression

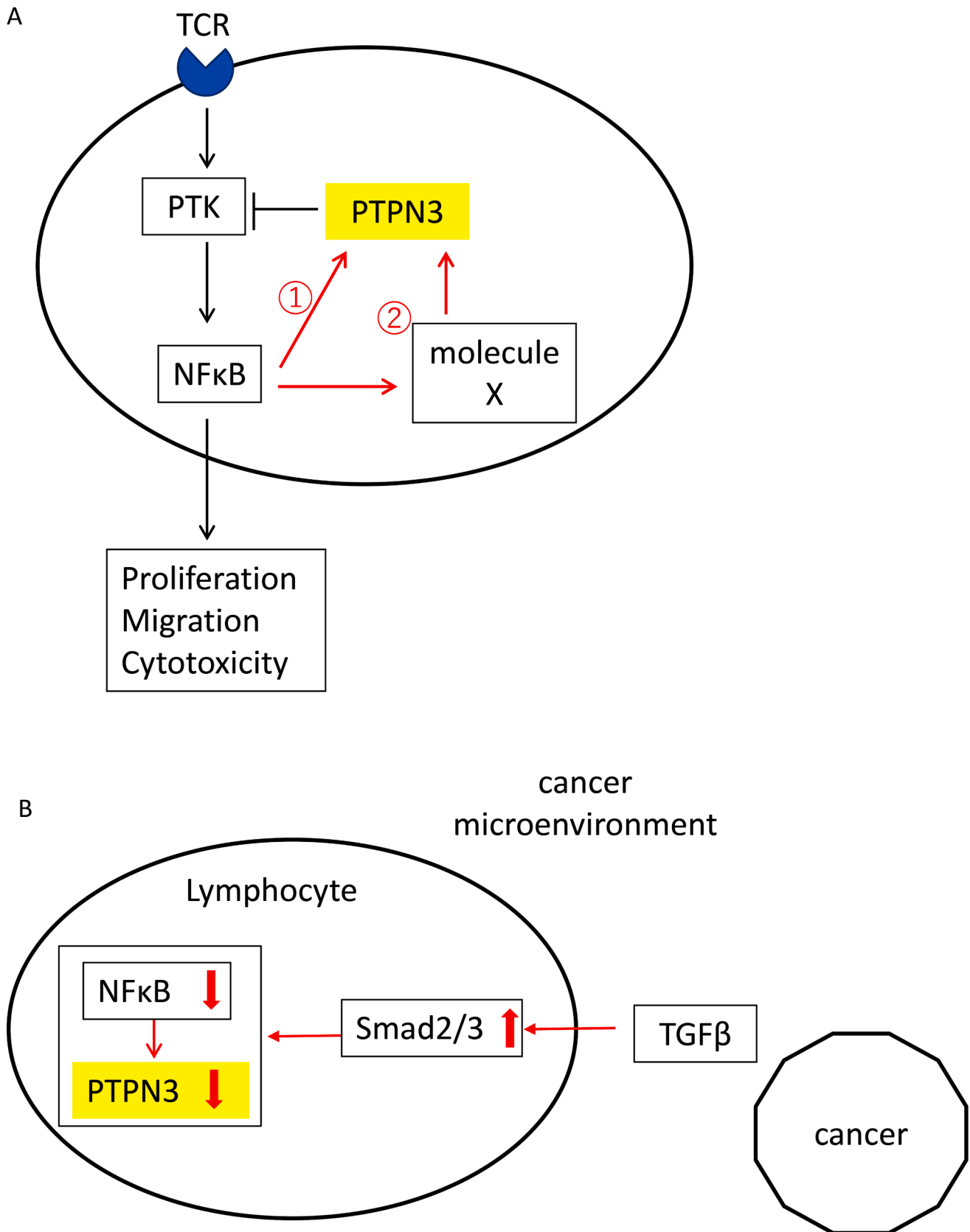
Our results suggest that NF $\kappa$ B is involved in the expression of PTPN3. To verify whether NF $\kappa$ B is directly involved in the expression of PTPN3, we performed luciferase assays. Using the Ensembl Genome Browser, we identified the transcription start site (TSS) of the PTPN3 gene (accession number NM\_002829.4) and obtained all PTPN3 gene sequences of Human GRCh38/hg38 using the UCSC Genome Browser. The PTPN3

gene sequences and the gene sequences around the TSS were compared by ApE (A plasmid Editor). Several predicted NF $\kappa$ B binding sites (GGGRNYYYCC) were observed around the TSS (Fig. 4A). PTPN3 promoter regions A and B, which included the predicted NF $\kappa$ B binding regions, were created as described in Methods (Fig. 4B). These regions were cloned into the pGL3-Promoter vector and designated as pGL3 (PTPN3-A) or pGL3 (PTPN3-B).

We next performed luciferase assays and co-transfected 293 T cells with the reporter gene construct, pHV-SV40, and specific expression plasmids (pCMV4-p65 expression plasmid or pCMV4-control plasmid) (Fig. 4C). Luciferase activity of the pGL3 (PTPN3-B) vector was increased compared with pGL3-Promoter empty vector, suggesting the possibility of the region having a cis-element (Fig. 4D). However, further luciferase activity was not observed with overexpression of NF $\kappa$ B. To eliminate the possibility that overexpressed NF $\kappa$ B did not act as trans-element due to a sufficient amount of NF $\kappa$ B present in 293 T cells, we also measured luciferase activity in 293 T cells with suppressed endogenous NF $\kappa$ B (Fig. 4E). However, similar results were obtained in cells with NF $\kappa$ B suppression (Fig. 4F). The result suggests that NF $\kappa$ B may be indirectly, but not directly, involved in regulating PTPN3 expression or that there may be a region that acts as an enhancer of NF $\kappa$ B binding other than the examined region.

### 3.5. TGF $\beta$ was involved in NF $\kappa$ B activation and PTPN3 expression through SMAD2/3 pathway in the cancer microenvironment

Previous studies have shown that activated lymphocytes work in the cancer microenvironment [15,16]. Therefore, we next examined changes in PTPN3 expression and NF $\kappa$ B activity of activated lymphocytes in the cancer microenvironment. First, the effect of cancer cells on PTPN3 expression in activated lymphocytes was examined by adding pancreatic cancer cell culture supernatant to activated lymphocytes. Inhibition of PTPN3 expression was observed by addition of cancer cell culture supernatant from various pancreatic cancer cell lines (Fig. 5A, B). However, we did not observe huge changes in NF $\kappa$ B (Fig. 5C). Cancer cells have been shown to release immunosuppressive cytokines, which affect immunity. Therefore, we next added TGF $\beta$  [17] or IL10, well-known immunosuppressive cytokines, to activated lymphocytes to examine the effects on PTPN3. Inhibition of PTPN3 expression was observed upon TGF $\beta$  addition, while no changes were observed in response to IL-10 (Fig. 5D), and pZAP70 and pSRC (pLCK) increased with TGF $\beta$  (Fig. 5D, E). We confirmed that TGF $\beta$  was detectable at high levels in the cancer cell culture supernatant (Fig. 5F). In addition, the contribution of TGF $\beta$  to the expression of PTPN3 was evaluated using TGF $\beta$  neutralizing antibody. Decrease of PTPN3 expression by cancer cell culture supernatant was abrogated by the addition of TGF $\beta$  neutralizing antibody (Fig. 5G). These results strongly suggest that TGF $\beta$  is involved with the expression of PTPN3. We also found that TGF $\beta$  decreased cytotoxic activity (Fig. 5H) and proliferation (Fig. 5I) and reduced NF $\kappa$ B activity (Fig. 5J) in activated lymphocytes. To know how



**Fig. 6.** A schematic illustration of the model. (A) NFκB enhances PTPN3 expression during the lymphocyte activation stage. We think 2 possibilities. One is that there may be a region that plays as an enhancer of NFκB binding other than the examined region (pathway ①). Another one is that a molecule X that is activated by NFκB may contribute to the expression of PTPN3 (pathway ②). (B) TGFβ reduces NFκB activation and PTPN3 expression through SMAD2/3 pathway in the cancer microenvironment.

PTPN3 is regulated by TGF $\beta$ , the contribution of SMAD2/3 that is activated by TGF $\beta$  stimulation [18,19] was investigated. Phosphorylated SMAD2/3 augmented by TGF $\beta$  treatment in activated lymphocytes (Fig. 5K). These results suggest that TGF $\beta$  produced by cancer cells may be involved in regulating NF $\kappa$ B activation and PTPN3 expression through SMAD2/3 pathway.

#### 4. Discussion

Here we demonstrate that the transcription factor NF $\kappa$ B is involved in the enhanced PTPN3 expression in activated lymphocytes. The PTPN3 gene is located on chromosome 9 (9q31.3 190375466-109498313) and is roughly 120 kb in length. Because the genomic region affecting promoter activity is often present around the TSS region, we cloned a construct containing the region around the TSS into a luciferase expression vector. Our results from this assay did not demonstrate a direct involvement of NF $\kappa$ B in PTPN3 expression. We suspect there may be a region that acts as an enhancer of NF $\kappa$ B binding other than the examined region or that several transcription factors may also be involved in the expression of PTPN3. We also do not exclude the possibility that NF $\kappa$ B may not be directly involved in PTPN3 expression. Future studies should verify NF $\kappa$ B binding sites throughout the PTPN3 gene region and examine other transcription factors that may be involved in the activation of lymphocytes.

In previous studies, suppression of PTPN3 led to enhanced tyrosine kinase activity and increased the functions of lymphocytes [7]. However, in this study, both PTPN3 and tyrosine kinase activity were decreased upon the suppression of NF $\kappa$ B. We do not have the definite answer about how NF $\kappa$ B regulates phosphorylation of ZAP70 at moment. We think that protein tyrosine kinase activity may concurrently change with PTPN3 expression to keep homeostasis in the activated lymphocytes. When NF $\kappa$ B is inhibited in activated lymphocytes, activation of lymphocytes as well as PTPN3 expression decreased in our results (Fig. 3). Whether NF $\kappa$ B is involved in phosphorylation of ZAP70 directly or indirectly will be the next direction of the experiment. NF $\kappa$ B acts as a transcription factor for various genes, regulating not only the expression of genes that are important for activation of lymphocytes, but also the expression of genes associated with negative feedback that regulates activation of lymphocytes [20]. PTPN3 may be one of the genes whose expression is regulated by NF $\kappa$ B and thereby regulates lymphocyte activation. In addition to TCR and CD28, IL-1, TNF $\alpha$ , 4-1BB (CD137), and other factors are also involved in NF $\kappa$ B activation, so future studies should analyze other factors that regulate the NF $\kappa$ B pathway.

The anti-tumor effects of cancer-infiltrated lymphocytes are important in cancer immunotherapy. Recently, research has been focused on the cancer microenvironment that is formed in the local area of tumors. For example, we found that in the hypoxic tumor environment, the function of activated lymphocytes and dendritic cells may be suppressed and that PD-L1 expression is upregulated [9,21]. Cytokine production is also an important factor in the cancer microenvironment. In this study, we found that TGF $\beta$  suppressed PTPN3 expression. Previous studies showed that TGF $\beta$  is an important immunosuppressive factor related to regulatory T cell induction, cancer fibrosis, and endothelial mesenchymal transition [22–24]. Future studies should examine the relationship between these factors and PTPN3 function. We suspect that PTPN3 may be involved in the suppression of lymphocyte activation and maintain homeostasis in association with PTK expression. NF $\kappa$ B is activated by signals from PTK, and the observation that the expression of PTPN3 is enhanced by NF $\kappa$ B is consistent with this finding. However, various molecules and cytokines act together on the immune system, so when suppressing or activating a single molecule, the expected result does not always occur. For example, in this study, PTPN3 was suppressed by TGF $\beta$  and pZAP70 and pSRC expressions were enhanced by the suppression of PTPN3 (Fig. 5E). PTK activation is involved in lymphocyte function enhancement, but addition of TGF $\beta$  suppressed

lymphocyte function. Downstream of pZAP70 and pSRC signaling pathway, TGF $\beta$  may suppress factors involved in lymphocyte activation. A previous study reported that TGF $\beta$  down-regulates the gene expression of CTL-mediated cytotoxic effector molecules such as perforin, granzymeA, granzymeB, IFN- $\gamma$ , and CD95L (FASL) [25], which are not involved in the TCR pathway. Therefore, the effector function of lymphocytes may be suppressed by these other pathways.

Fig. 6 shows the proposed model for the findings of this study. We think two possibilities of PTPN3 expression through lymphocyte-activated process as shown in Fig. 6A. One possibility is that there may be a region that acts as an enhancer of NF $\kappa$ B binding other than the examined region (pathway ). Another possibility is that a molecule X that is activated by NF $\kappa$ B may contribute to the expression of PTPN3 (pathway@). In cancer local site, TGF- $\beta$  may decrease NF $\kappa$ B activation and PTPN3 expression through SMAD2/3 pathway (Fig. 6B).

PTPN3 was previously identified as an immune checkpoint molecule, and PTPN3 inhibitory therapy may become a novel cancer therapy as a novel non-antibody type immune checkpoint inhibitor in the future. In this respect, the results of this study, which show that NF $\kappa$ B and TGF $\beta$  are involved in the mechanism underlying PTPN3 expression in activated lymphocytes, are expected to provide significant implications for the improvement of existing immunotherapeutic effects and the development of new cancer immunotherapies.

#### Funding

This study was supported by the Japan Society for the Promotion of Science KAKENHI Grant [grant numbers 19K09124, 19K09047, and 20K09180].

#### Author contributions

Kazunori Nakayama was involved in the analysis of all experiments. Akiko Fujimura and Yuichi Fujimoto were involved in the acquisition and analysis of data. Akira Imaizumi and Makoto Kawamoto were involved in the gene transfection. Yasuhiro Oyama, Shu Ichimiya and Satoko Koga were involved in the interpretation of data. Kinichi Nakashima, Masafumi Nakamura, and Hideya Onishi were involved in the design of the work.

#### Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

#### Acknowledgements

We thank Ms. Emi Onishi for skillful technical assistance. We thank the technical assistance from The Research Support Center, Research Center for Human Disease Modeling, Kyushu University Graduate School of Medical Sciences. We thank Edanz Group (<https://en-author-services.edanzgroup.com/>) for editing a draft of this manuscript. Dr. Takashi Morisaki kindly gives us calcein staining reagent.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cellimm.2020.104237>.

#### References

- [1] P.C. Tumeh, C.L. Harview, J.H. Yearley, I.P. Shintaku, E.J. Taylor, L. Robert, B. Chmielowski, M. Spasic, G. Henry, V. Ciobanu, A.N. West, M. Carmona, C. Kivork, E. Seja, G. Cherry, A.J. Gutierrez, T.R. Grogan, C. Mateus, G. Tomasic, J. A. Glaspy, R.O. Emerson, H. Robins, R.H. Pierce, D.A. Elashoff, C. Robert, A. Ribas, PD-1 blockade induces responses by inhibiting adaptive immune resistance, *Nature* 515 (7528) (2014) 568–571.

- [2] H. Borghaei, L. Paz-Ares, L. Horn, D.R. Spigel, M. Steins, N.E. Ready, L.Q. Chow, E. E. Vokes, E. Felip, E. Holgado, F. Barlesi, M. Kohlhäufel, O. Arrieta, M.A. Burgio, J. Fayette, H. Lena, E. Poddubskaya, D.E. Gerber, S.N. Gettinger, C.M. Rudin, N. Rizvi, L. Crino, G.R. Blumenschein Jr., S.J. Antonia, C. Dorange, C.T. Harbison, F. Graf Finckenstein, J.R. Brahmer, Nivolumab versus docetaxel in advanced non-squamous non-small-cell lung cancer, *N. Engl. J. Med.* 373 (17) (2015) 1627–1639.
- [3] S.E. Barnes, Y. Wang, L. Chen, L.L. Molinero, T.F. Gajewski, C. Evaristo, M. L. Alegre, T cell-NFκB activation is required for tumor control in vivo, *J. Immunother. Cancer* 3 (1) (2015) 1.
- [4] H. Onishi, A. Kiyota, N. Koya, H. Tanaka, M. Umebayashi, M. Katano, T. Morisaki, Random migration contributes to cytotoxicity of activated CD8+ T-lymphocytes but not NK cells, *Anticancer Res.* 34 (8) (2014) 3947–3956.
- [5] S.E. Barnes, Y. Wang, L. Chen, L.L. Molinero, T.F. Gajewski, C. Evaristo, M. L. Alegre, T cell antigen receptor signaling, *Eur. J. Immunol.* 30 (5) (2000) 1318–1325.
- [6] N.K. Tonks, B.G. Neel, Combinatorial control of the specificity of protein tyrosine phosphatases, *Curr. Opin. Cell Biol.* 13 (2) (2001) 182–195.
- [7] A. Fujimura, K. Nakayama, A. Imaizumi, M. Kawamoto, Y. Oyama, S. Ichimiya, M. Umebayashi, N. Koya, T. Morisaki, T. Nakagawa, H. Onishi, PTPN3 expressed in activated T lymphocytes is a candidate for a non-antibody-type immune checkpoint inhibitor, *Cancer Immunol. Immunother.* 68 (10) (2019) 1649–1660.
- [8] J.E. Smith-Garvin, G.A. Koretzky, M.S. Jordan, T cell activation, *Annu. Rev. Immunol.* 27 (2009) 591–619.
- [9] T. Ogino, H. Onishi, H. Suzuki, T. Morisaki, M. Tanaka, M. Katano, Inclusive estimation of complex antigen presentation functions of monocyte-derived dendritic cells differentiated under normoxia and hypoxia conditions, *Cancer Immunol. Immunother.* 61 (3) (2012) 409–424.
- [10] K. Suyama, H. Onishi, A. Imaizumi, K. Shinkai, M. Umebayashi, M. Kubo, Y. Mizuuchi, Y. Oda, M. Tanaka, M. Nakamura, M. Katano, CD24 suppresses malignant phenotype by downregulation of SHH transcription through STAT1 inhibition in breast cancer cells, *Cancer Lett.* 374 (1) (2016) 44–53.
- [11] H. Onishi, T. Morisaki, A. Kiyota, N. Koya, H. Tanaka, M. Umebayashi, M. Katano, The Hedgehog inhibitor cyclopamine impairs the benefits of immunotherapy with activated T and NK lymphocytes derived from patients with advanced cancer, *Cancer Immunol. Immunother.* 62 (6) (2013) 1029–1039.
- [12] S. Martínez-Martínez, P. Gómez del Arco, A.L. Armesilla, J. Aramburu, C. Luo, A. Rao, J.M. Redondo, Blockade of T-cell activation by dithiocarbamates involves novel mechanisms of inhibition of nuclear factor of activated T cells, *Mol. Cell. Biol.* 17 (11) (1997) 6437–6447.
- [13] K. Matsumoto, T. Morisaki, H. Kuroki, M. Kubo, H. Onishi, K. Nakamura, C. Nakahara, H. Kuga, E. Baba, M. Nakamura, K. Hirata, M. Tanaka, M. Katano, Exosomes secreted from monocyte-derived dendritic cells support in vitro naive CD4+ T cell survival through NFκB activation, *Cell. Immunol.* 231 (1–2) (2004) 20–29.
- [14] I. Mattioli, A. Sebald, C. Bucher, R.P. Charles, H. Nakano, T. Doi, M. Kracht, M. L. Schmitz, Transient and selective NF-κB p65 serine 536 phosphorylation induced by T cell costimulation is mediated by I kappa B kinase beta and controls the kinetics of p65 nuclear import, *J. Immunol.* 172 (10) (2004) 6336–6344.
- [15] G. Pruneri, A. Vingiani, C. Denkert, Tumor infiltrating lymphocytes in early breast cancer, *Breast* 37 (2018) 207–214.
- [16] MacLean Hall, Hao Liu, Mokenge Malafa, Barbara Centeno, Pamela J. Hodul, José Pimiento, Shari Pilon-Thomas, Amod A. Sarnaik, Expansion of tumor-infiltrating lymphocytes (TIL) from human pancreatic tumors, *J. Immunother. Cancer* 61 (4) (2016) 5.
- [17] M. Ahmadzadeh, S.A. Rosenberg, TGF-beta1 attenuates the acquisition and expression of effector function by tumor antigen-specific human memory CD8 T cells, *J. Immunol.* 174 (9) (2005) 5215–5223.
- [18] J.-S. Delisle, M. Giroux, G. Boucher, J.-R. Landry, M.-P. Hardy, S. Lemieux, R. G. Jones, B.T. Wilhelm, C. Perreault, The TGF-β-Smad3 pathway inhibits CD28-dependent cell growth and proliferation of CD4 T cells, *Genes Immun.* 14 (2) (2013) 115–126.
- [19] Philip J. Lucas, Nicole McNeil, Eva Hilgenfeld, Baishakhi Choudhury, Seong-Jin Kim, Michael A. Eckhaus, Thomas Ried, Ronald E. Gress, Transforming growth factor-β pathway serves as a primary tumor suppressor in CD8+ T cell tumorigenesis, *Cancer Res.* 64 (2004) 6524–6529.
- [20] S. Paul, B.C. Schaefer, A new look at T cell receptor signaling to nuclear factor-κB, *Trends Immunol.* 34 (6) (2013) 269–281.
- [21] H. Onishi, A. Fujimura, Y. Oyama, A. Yamasaki, A. Imaizumi, M. Kawamoto, M. Katano, M. Umebayashi, T. Morisaki, Hedgehog signaling regulates PDL-1 expression in cancer cells to induce anti-tumor activity by activated lymphocytes, *Cell. Immunol.* 310 (2016) 199–204.
- [22] W. Chen, W. Jin, N. Hardegen, K.J. Lei, L. Li, N. Marinos, G. McGrady, S.M. Wahl, Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3, *J. Exp. Med.* 198 (12) (2003) 1875–1886.
- [23] D.R. Principe, B. DeCant, E. Mascariñas, E.A. Wayne, A.M. Diaz, N. Akagi, R. Hwang, B. Pasche, D.W. Dawson, D. Fang, D.J. Bentrem, H.G. Munshi, B. Jung, P.J. Grippo, TGFβ signaling in the pancreatic tumor microenvironment promotes fibrosis and immune evasion to facilitate tumorigenesis, *Cancer Res.* 76 (9) (2016) 2525–2539.
- [24] J. Massagué, TGFbeta in cancer, *Cell* 134 (2) (2008) 215–230.
- [25] D.A. Thomas, J. Massagué, TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance, *Cancer Cell* 8 (5) (2005) 369–380.